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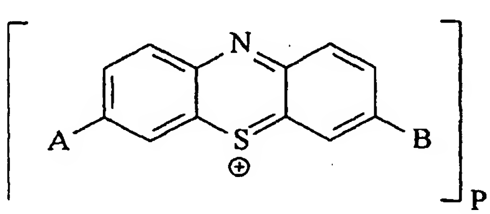
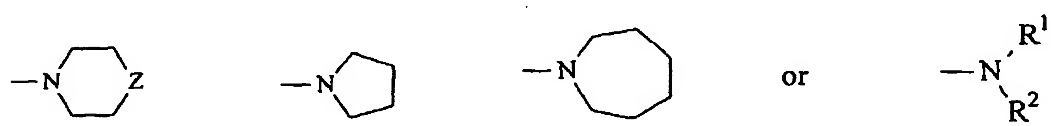
(54) Title: BIOLOGICALLY ACTIVE METHYLENE BLUE DERIVATIVES



X^{P-} (I)



(57) Abstract: The present invention relates to a phenothiazinium compound of Formula (I), wherein A and B are each independently (a) or (b) in which Z is CH_2, O, S, SO_2, NH, NCH_3, NC_2H_5, NCH_2CH_2OH, or $NCOCH_3$ and R^1 and R^2 are each independently linear or branched $C_nH_{2n}Y$, where n is 1-6, Y is H, F, Cl, Br, I, OH, OCH_3, OC_2H_5, OC_3H_7, CN or $OCOCH_3$, and where X^{P-} is a counteranion and P is 1, 2 or 3 but not including a compound where A and B are both either $-N(CH_3)_2$ or $-N(CH_2CH_3)_2$. The invention also relates to use of the compound of the invention as a PDT agent or a photodiagnostic agent.

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BIOLOGICALLY ACTIVE METHYLENE BLUE DERIVATIVES

FIELD OF THE INVENTION

This invention relates to biologically active photosensitisers which are strongly photocytotoxic and have application in the areas of photodynamic therapy (PDT), as well as for the diagnosis and detection of medical conditions and related uses in photochemical internalisation, in the production of cancer vaccines, in the treatment of microbial infections and in photodisinfection or photosterilisation.

BACKGROUND TO THE INVENTION

It is known that certain organic compounds ("*photosensitisers*") can induce cell death by absorption of light in the presence of oxygen. The cytotoxic effect involves *Type I* and/or *Type II* photooxidation. Such photosensitisers find use in the treatment of cancer and other diseases or infections with light (*photodynamic therapy)* and in the sterilisation (including disinfection) of surfaces and fluids by the light-induced destruction of microbes. In this context, the term sterilisation is taken to mean the reduction or elimination of microbes in a particular situation.

It is also known that certain coloured phenothiazinium compounds, (*e.g.* methylene blue) can take part in Type I and Type II photooxidation processes, but compounds of this type thus far have proved unsuitable or of low efficacy as sensitisers for photodynamic therapy, or have shown low photochemical antimicrobial activity.

For application in PDT, a good sensitiser must have at least some and preferably all of the following properties. Most importantly, it should cause the destruction of target cells (for example tumour cells or bacterial cells) efficiently on exposure to light (preferably wavelengths *ca.* 600 - 800 nm). The PDT treatment using the photosensitiser should show a high degree of selectivity between target and normal

tissues. The sensitiser should have relatively little dark toxicity and it should cause little or no skin photosensitivity in the patient.

For applications in photosterilisation, a good sensitiser must show a strong phototoxic effect in a wide range of microrganisms, ideally using ambient light, and should not photobleach readily.

In oncology, several different types of photosensitiser have been used to treat both solid tumours and thin tumours of hollow organs such as the oesophagus and bladder. However, the use of these photosensitisers has been restricted partly because of lack of selectivity between tumour and healthy tissue and partly because of the prolonged skin photosensitivity which can be caused. There is a need for new photosensitisers which cause little or no skin photosensitivity and which selectively destroy tumour cells.

Although PDT has previously been used in the treatment of tumours, it has not yet been used against infections caused by bacteria and other microorganisms. The use of antibiotics to treat bacterial infections is becoming challenging due to the increasing resistance of many bacterial species to commonly used antibiotics, such as tetracyclines and beta-lactams. Hospital-acquired antibiotic resistant infections such as MRSA are especially problematic. Photodynamic antibacterial treatment is a promising alternative to antibiotics for local treatment.

When developing antibacterial agents a major problem which must be overcome is the uptake of the drug into the bacterial cell. Gram negative and Gram positive bacteria differ in the composition of their outer surface and respond differently to antimicrobial agents, especially in terms of uptake. Due to the high negatively charged surface of Gram negative bacteria they are relatively impermeable to neutral or anionic drugs, including most commonly used photosensitisers. Development of antimicrobial photosensitisers which are effective against Gram negative bacteria, as

well as Gram positive bacteria would be highly beneficial to replace commonly used antibiotics and drugs which are becoming increasingly ineffective due to resistance.

A number of different types of photosensitiser have been investigated in bacteria. These include phenothiazinium compounds, phthalocyanines, chlorins and naturally occurring photosensitisers. For uptake into Gram negative bacteria, it is accepted that the cationic derivatives are the most effective.

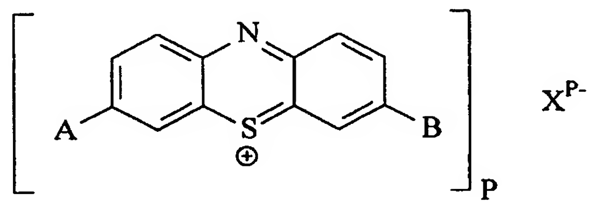
Phenothiazinium compounds are blue dyes with maximum absorption at wavelengths between 600-700 nm. They have been studied for their non-photodynamic antibacterial properties but few apart from methylene blue and toluidine blue have been investigated photodynamically.

Wainwright *et al* (1998) investigated the effect of a series of phenothiazinium methylene blue derivatives in tumour cell lines and bacteria. New methylene blue (NMB) and di methyl methylene blue (DMMB) were effective at inactivating *MRSA* and were shown to be more effective photosensitisers than methylene blue when acting on pigmented melanoma cell lines. Wagner *et al* (1998) studied these dyes and in addition a hydrophobic derivative for the inactivation of enveloped viruses.

The precise mode of antibacterial action of methylene blue is unknown, but one hypothesis is that because of its stereochemistry it can intercalate into DNA, and that photodynamic action causes DNA damage. Methylene blue itself has been shown to be ineffective as an anti-tumour agent. In addition, methylene blue is known to be susceptible to photobleaching, which could be a serious disadvantage in some applications. Because of the recognised limitations of methylene blue, both anti-tumour PDT and antimicrobial PDT would benefit from development of new phenothiazine-based photosensitisers.

STATEMENTS OF THE INVENTION

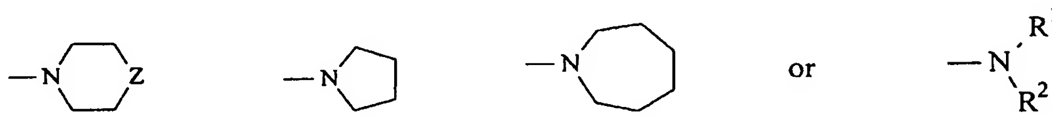
According the present invention there is provided a phenothiazinium compound of Formula (I):



(I)

wherein:

A and B are each independently



in which Z is CH_2, O, S, SO_2, NH, NCH_3, NC_2H_5, NCH_2CH_2OH, or $NCOCH_3$ and R^1 and R^2 are each independently linear or branched $C_nH_{2n}Y$, where n is 1-6, Y is H, F, Cl, Br, I, OH, OCH_3, OC_2H_5, OC_3H_7, CN or $OCOCH_3$,

and where X^{P-} is a counteranion and P is 1, 2 or 3

but not including a compound where A and B are both either $-N(CH_3)_2$ or $-N(CH_2CH_3)_2$.

Preferably the counteranion is selected from any of Cl^-, Br^-, I^-, F^-, NO_3^-, HSO_4^-, $CH_3CO_2^-$, or is a dianion, namely, SO_4^{2-} or HPO_4^{2-}, or trianion, namely PO_4^{3-}.

Preferably A and B are the same and both R^1 and R^2 are n-propyl, n-butyl or n-pentyl.

The compound may be used against microorganisms. Preferably the compound is used against against bacteria. More preferably the compound is used against antibiotic resistant bacteria.

In one embodiment of the invention the compound is for use as a PDT agent or a photodiagnostic agent.

Furthermore, the present invention provides a conjugate or composite formed between a compound of Formula I and a polymer. The term composite as used herein refers to the situation wherein a compound of the invention is embedded in a polymer or physically occluded within or adsorbed onto a matrix or substrate. The polymer may be a biological polymer such as a peptide or a protein. Preferred polymers include those having anhydride and/or ester groups.

In addition, the present invention provides a compound formed by the reaction between a compound of Formula I and a chlorotriazine derivative. The chlorotriazine derivative may be a polymer having chlorotriazine groups attached thereto.

In one embodiment compounds of the present invention are used as a medicament.

The compounds of the present invention are useful as photosensitising drugs for PDT of conditions where treatment requires removal of unwanted tissue or cells such as cancer, precancerous disease, ophthalmic disease, vascular disease, autoimmune disease, and proliferative conditions of the skin and other organs. Specific and unpredicted advantages of these materials relate to their ability to be photoactive against target tissues at different times after systemic administration (depending upon the particular sensitiser used) and therefore their ability to be targeted to the vasculature or tumour cells (for example) directly. They also have a low tendency to sensitise skin to ambient light when administered systemically and a low tendency to colour skin.

The compounds may also be used in PDT as photoactivatable antimicrobial treatments for skin and other local infections, for sterilisation of burn wounds and other lesions, for sterilisation of both recipient tissue and donated tissue during organ including skin transplantation and for the treatment of dental microbial disease.

The said compounds are also useful as photoactivatable antimicrobial agents for general sterilisation of surfaces and fluids. Specific advantages of these compounds over existing known antimicrobial photosensitisers are their high photocytotoxicity at low light levels, combined with a low tendency to undergo photobleaching.

Furthermore, compounds of the present invention have the advantage, compared with other phenothiazinium photosensitisers, that they do not, in carrying out their cell-destroying activity, target the nucleus of the cell so that there is a much lower risk of the cells undergoing mutagenic transformations.

The present invention also provides compositions comprising a compound of the present invention together with a diluent or excipient.

Examples of uses of the compounds of the present invention are as photosensitising drugs for PDT to treat Barrett's oesophagus and cervical intraepithelial neoplasia (pre-cancerous diseases), bladder cancer and colon cancer, macular degeneration (an ophthalmological disease), vascular problems such as cardiovascular disease, arteriosclerosis and restenosis and autoimmune diseases such as rheumatoid arthritis, skin diseases such as psoriasis, acne and excema and other benign conditions such as endometriosis and menorrhagia. The compounds may also be used as anti-microbial treatments for skin and wound infections, other local infections as well as in the treatment of dental bacterial disease. The compounds may also be used in photochemical internalisation (the use of photosensitisers to assist the uptake and subcellular localisation of drugs) through their photosensitising properties and in non-therapeutic uses such as in photodiagnosis through their fluorescence properties. The latter approach takes advantage of the fact that the photosensitiser concentrates

more in tumours than in surrounding healthy tissue and when induced to fluoresce (by application of blue light), the tumour fluoresces more strongly than the surrounding tissue.

The compounds may be used as a photoactivated antimicrobial agents for sterilisation of surfaces and fluids.

Accordingly, the present invention provides a method of treatment for cancer and other human or animal diseases through systemic or local administration of the photosensitiser, followed by application of light of an appropriate dose and wavelength or wavelength range.

Preferably the compound administered to a subject in need of treatment is that according to formula (I), where R^1 and R^2 are n-propyl and said light exposure is given up to 10 minutes from the point of drug administration.

In a preferred embodiment of the invention, light exposure is given within 1 minute of drug administration.

More preferably light exposure is give at the point of drug administration.

Alternatively the compound administered is that according to formula (I), where R^1 and R^2 are n-pentyl and said light exposure is given at longer times from the point of drug administration.

Accordingly, the present invention provides a method of treatment of microbial infections, burn wounds and other lesions and of dental bacterial disease, the method comprising systemic administration or applying to the area to be treated (for example by a spray) a therapeutically effective amount of a compound of the present invention and exposing said area to light to render active said compound.

Preferably the method comprises the step of administering a compound according to formula (I) where R^1 and R^2 are n-butyl.

Furthermore, the present invention also provides a method of sterilising a surface or a fluid comprising applying the compound of the present invention to said surface or fluid and activating said compound by means of light.

Appropriate compounds of the present invention may be attached to polymeric surfaces, permanently by covalent bonds or reversibly by intermolecular interactions, thus affording a surface that can be sterilised whenever required by the application of light. This would be useful, for example, with intravenous lines in patients and in sutures and catheters and intravenous lines, where maintaining long-term sterility of the relevant surfaces is problematical. The resistance of the compounds to photobleaching is an advantage in such applications, where prolonged stability of the chromophore is required.

Accordingly the present invention also provides an article having at least one surface to which is attached a compound of the present invention.

Preferably the article is a medical device such as a venous, urinary or balloon catheter, suture, orthopaedic or artifical implant, heart valve, surgical screw or pin, pacemaker lead, feeding or breathing tube, vascular stent, intraocular lens, or small joint replacement. The article may also be of use in wound care and for packaging materials for medical use, for example, materials for medical equipment

A compound of the present invention may be applied to walls, floors and ceilings of hospitals, clinical surfaces such as operating tables, abattoirs and clean rooms in scientific laboratories. Fibres may be converted into woven, knitted or non-woven textile articles such as cleaning cloths, wipes, surgical gowns, bed linen, wound dressings and bandages.

Alternatively the article is one for use in the food and beverage industry and may be an item of packaging, a wrapper or storage carton or a piece of processing equipment. The article may be a refrigerator, vending machine, ice making machine, a piece of restaurant equipment or other kitchen appliance.

The present invention relates to phenothiazium sensitizers which show an unexpected and pronounced dependence of their photobiological properties *in vitro* and *in vivo* on the size and hydrophobic character of substituents on the terminal amino groups. By careful selection of such structural features, photosensitisers with distinct advantages over existing materials are provided. Accordingly compounds of the present invention overcome the problems of the prior art by providing the following advantages in the field of oncology and in their antimicrobial effects:

Advantages in oncology

- Extremely strong photoactivity when compared with methylene and ethylene blue.

- Low absorption of light in the UV/blue region. This results in a lower propensity of the compounds to skin photosensitivity.

- Rapid skin clearance.

- High selectivity for tumours.

- Low dark toxicity.

- Low potential for DNA damage when compared with methylene blue.

- Very short drug-to-light time interval compared with existing PDT drugs.

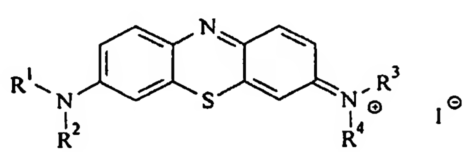
Antimicrobial advantages

- Highly effective in deactivating a wide range of microorganisms, including Gram positive and Gram negative bacteria, MRSA and fungal infection.
- Active against quiescent bacteria.
- High selectivity for microorganisms with minimum damage to host tissue.
- Unexpectedly low level of photobleaching.

DETAILED DESCRIPTION OF THE INVENTION

Compounds examined

The phenothiazinium compounds used in this study were synthesised in Leeds University Department of Colour Chemistry by J. Griffiths. They included the following:



$R^1 - R^4 = $ n- C_3H_7 : tetra-n-propyl

$R^1 - R^4 = $ n- C_4H_9 : tetra-n-butyl

$R^1 - R^4 = $ n- C_5H_{11} : tetra-n-pentyl

$R^1 - R^4 = $ n- C_6H_{13} : tetra-n-hexyl

Methylene blue ($R^1 - R^4 = $ n-CH_3) and ethylene blue ($R^1 - R^4 = $ n-C_2H_5) were also examined for comparative purposes.

Stock solutions of photosensitisers were made up in water and/or DMSO and stored in the dark until required. Test solutions were made up in buffer or solvent or biological medium as required.

Spectral and physical properties of the phenothiazinium compounds

Spectral data of the phenothiazinium compounds in water and in methanol (Table 1), show that all of the compounds have absorption peaks in the 650 – 700 nm region, but that there is considerable variability in the precise peak position. The phenothiazinium compounds with longer alkyl chains have absorption peaks at longer wavelengths and the peak positions in general are at longer wavelengths in water compared with methanol. These differences probably reflect the aggregation state of the photosensitisers .

The octanol-water partition coefficients (*log p*) for the various photosensitisers are also shown in Table 1, where

$$log\, p \;=\; (\text{mg/ml in octanol}) / (\text{mg/ml in buffer})$$

Table 1 Spectral and physical properties of the phenothiazinium compounds

R	Wavelength max in water (nm)	Wavelength max in methanol (nm)	Ex.Max (nm)	Em.Max (nm)	*logP*
1	667	656	640	690	-1.0
2	673	661	670	690	+0.2
3	679	665	680	690	+1.1
4	682	668	680	695	+1.1
5	685	669	680	700	+1.7
6	699	669	675	700	+1.3

Ex.Max is the fluorescence excitation wavelength maximum and Em.Max is the fluorescence emission wavelength maximum

The octanol buffer partition coefficient (*logP)* determines the lipophilicity of the drug. As might be expected, the lipophilicity increases with increasing value of R,

but it should be noted that even for higher values of R, the compounds remain soluble in biological media.

Phenothiazinium derivatives as PDT agents for mammalian cells and tumours

The phenothiazinium derivatives were assessed for PDT efficacy in a series of mammalian cells in culture. RIF-1 murine fibrosarcoma cells were studied, using the MTT assay to assess remaining cell viability following PDT. The data from a series of experiments are summarised in Table 2, which shows the LD_{50} values (concentration of photosensitiser needed to kill half of the cells under the conditions used) for four of the phenothiazine derivatives. Also shown for comparison are the data for methylene blue and ethylene blue. Some of these compounds are also able to kill cells in the dark and Table 2 also shows the ratio to LD_{50} for dark only controls. It may be seen from Table 2 that the tetra-n-pentyl derivative, the tetra-n-butyl derivative and the tetra-n-propyl derivative are all efficient PDT agents under these conditions, being much more active than methylene blue or ethylene blue. The most efficient is the tetra-n-propyl derivative. Also, it is clear that, while for methylene blue there is only a small ratio between the LD_{50} for dark and light toxicity, this ratio is much greater for the phenothiazinium derivatives.

The results illustrate the much increased photoactivity of these compounds, but also their relative lack of dark toxicity compared with methylene blue. This is a considerable advantage in therapeutic terms. Table 2 also shows the relative activity of the various derivatives in comparison with methylene blue and ethylene blue using a measure of their intrinsic ability to produce singlet oxygen. It may be seen that there is very little difference between the different compounds, showing that the marked differences observed in cells is almost entirely due to biological factors, the mechanisms of which are not yet known. Table 2 also shows the initial subcellular localisation of the different phenothiazinium compounds derivatives compared with that of methylene blue and ethylene blue, as well as any re-localisation which occurs following light administration. It is noteworthy that, whilst all of the derivatives initially localise in lysosomes, whilst methyene blue then relocalises to the nucleus

(with possible deleterious or mutagenic effects on DNA), the tetra-n-propyl, tetra-n-butyl, tetra-n-pentyl and tetra-n-hexyl derivatives relocalise to the mitochondria, which is a much better PDT target.

Table 3 shows LD_{50} values for some of the derivatives in a series of different cells in culture, representing different human tissues and cancers. It is clear that the tetra-n-propyl, the tetra-n-butyl and the tetra-n-pentyl derivatives are again highly active compared with methylene blue and that they are also active in all cell lines tested.

Several asymmetric phenothiazinium derivatives (where $R^1 = R^2 \neq R^3 = R^4$) have been prepared and tested in cells in culture. Several of these have proved superior photosensitisers to methylene blue, both in terms of absolute activity and in terms of the light to dark toxicity ratio. Sample data for these compounds are shown in Table 4.

Table 4. Chemical properties of the asymmetric phenothiazines in comparison to methylene blue and phototoxicity and dark toxicity in SiHa human cervical squamous cell carcinoma cells.

	λ_{max} in methanol (nm)	Singlet oxygen generation[1]	Log P	PDT LD_{50} (μM)[2]	Ratio dark : PDT LD_{50}[2]
Methylene blue	656	47	-1.0	18.7 ± 1.0	1
Di-n-butyl morpholino	661	29	+1.0	4.6 ± 2.0	7
Di-n-butyl diethanolamine	660	13	+1.3	1.7 ± 0.3	87
Di-n-pentyl diethanolamine	663	32	+1.1	0.43 ± 0.03	39

[1] % photo-oxidation of 1,3-diphenylisobenzofuran after 10 min red light illumination with 100mg/ml of the phenothiazinium compounds in 90% DMF : 10% water.

[2] Cells were incubated with the phenothiazinium compounds for 2h. For measurement of phototoxicity, cells were illuminated with 3J / cm^2 665nm light. Dark toxicity was measured in parallel. Cell survival was assessed after 48h using the sulforhodamine B (SRB) assay.

Figure 1 shows the anti-tumour photodynamic efficacy (% tumour necrosis) of symmetrically substituted thiazines of type (I). Female CBA/Gy mice with CaNT subcutaneous tumours were injected with a solution of the drug at a dose of 16.6 μmol per kilogram. They were treated with the optimum wavelength of light (determined in separated experiments) 1 hour after injection. The light source was a Patterson lamp with appropriate filters giving a bandwidth of 30 nm, and treatment was 60 J cm^{-2} at a rate of 50 mW cm^{-2}. It can be seen from Figure 1 that tumour response is very dependent on the nature of the alkyl groups, and the tetra-n-pentyl

and tetra-n-butyl derivatives were particularly effective compared with methylene blue.

Figure 2 shows the anti-tumour photodynamic efficacy (% tumour necrosis) for the tetra-n-propyl and tetra-n-pentyl derivatives as a function of the time interval between drug and light administration. These data show a quite unexpected difference between the two compounds. The tetra-n-propyl derivative is very active at very short drug-light time intervals (ie by giving light almost immediately after giving drug) whereas the tetra-n-pentyl derivative has very low activity at very short times, but much higher activity after 1 hour. The explanation for this finding is as yet unknown, but clearly these differential properties could be exploited for different applications. For example, the fast acting drug could be used for vascular treatments and the slower acting drug could be used for tumour cell treatments.

Figure 3 shows the relative skin photosensitivity caused by the tetra-n-butyl and tetra-n-pentyl derivatives, compared with polyhaematoporphyrin, PHP (equivalent to Photofrin). Photofrin is the current leading PDT agent for oncology, but has the major disadvantage of causing prolonged skin photosensitivity in patients. In this model, the skin photosensitivity is measured in terms of the increase in ear thickness 24 hours after exposure to drug and light. Figure 3 shows that, as expected, PHP shows a high level of skin photosensitivity, but the two phenothiazinium derivatives show little or no skin photosensitivity. These two derivatives also caused very little skin colouration after administration.

Photo-antimicrobial activity

General Methods

The methods given below are in respect of E coli, but were essentially the same for other bacteria studied ie *P.aeruginosa, S.aureus and MRSA.*

Standard preparation of bacteria

The standard protocol outlined below was modified as appropriate to study variation of various experimental parameters.

100ml of nutrient broth (0.5% yeast extract 1.0% tryptone w/v) was aseptically inoculated with E.coli strain DH5, and incubated in a shaking incubator overnight at 37°C. The incubator was set to 250 strokes per minute and a rotary motion of a 2.5cm circle. Following incubation 10ml of the culture was aseptically transferred to 200ml of nutrient broth, and grown in the shaking incubator detailed above until in mid log phase. Cells were collected by centrifugation (3000rpm, 10 minutes), and resuspended in 0.1M potassium phosphate buffer (pH7.0) and centrifuged again (3000rpm, 10 minutes). The supernatant was discarded and the pellet resuspended in 0.1M potassium phosphate buffer (pH7.0) to an absorbance of 0.85-0.90 at 650nm.

In the experiments involving illumination in nutrient media, bacterial cells were resuspended in nutrient media at this stage.

Bacterial cell inactivation experiments

25ml of the prepared cell suspension was incubated with 0.25mls of a 0.1mM stock solution of photosensitiser in a 250ml sterile foil covered conical flask. The suspension was incubated for 30 minutes in the dark in a 37°C shaking incubator at 250rpm. The suspension was irradiated by a 500W halogen lamp, from a distance of 75cm, for 60 minutes, the power of the lamp was 1.3mW/cm^2 giving 4.68J/cm^2 over the hour illumination. 50ml of the illuminated and nonilluminated samples of the suspension were removed and diluted in 0.1 M pH 7.0 potassium phosphate buffer. 50ml of the diluted suspension was then plated on nutrient agar (0.5% yeast extract, 1.0% tryptone, 2.0% agar w/v) and incubated overnight at 37°C to give a number of colony forming units between 30-300. Cell inactivation was then measured.

Control studies involving plating out of bacteria before and after the 30 minute incubation step with no phenothiazinium compounds but 0.25mls DMSO showed

no change in CFU/ml. Illumination of the bacterial culture alone with no phenothiazinium compounds but 0.25mls DMSO also demonstrated no change in CFU. For illumination in nutrient media, control tests showed a $\log_{10}$ increase in CFU/ml of 0.2 during the hour illumination.

Some irradiations were carried out with a laser, when the bacterial suspension was illuminated with a 665nm Ceramoptec diode array laser at 0.1W.

Determination of the effect of phenothiazinium compounds on bacterial cell growth
200ml of nutrient media (0.5% yeast extract 1.0% tryptone w/v) in foil covered 250ml conical flasks was aseptically innoculated with 10ml of a fully grown bacterial culture. In addition the media contained 1.0 ml of a 1mM stock solution of phenothiazinium compounds with a final concentration of 10µM, apart from the control which contained no phenothiazinium compounds but 1.0 ml DMSO.

The suspension was incubated at 37°C and 250rpm in a shaking incubator in the dark. 1ml samples were taken every hour for 6 hours and turbidity based on apparent optical density at 550nm caused by light scattering was measured. Control studies show this wavelength is out of the region of photosensitiser absorption. Following optical density readings the 1.0ml sample was spun in a MSE Micro-Centaur centrifuge (10 000*g* x 5 minutes) and the absorbance spectra of the supernatant read spectrophotometrically.

For the tetra-n-butyl derivative only, similar experiments were carried out where the bacteria were allowed to grow without photosensitiser for 3 hours, after which time the phenothiazinium compounds was added. Subsequent growth was monitored as a function of time, both for exposure to light and in the dark.

Preparation and light-induced inactivation of Candida albicans
For the experiments involving the yeast Candida albicans the organism was grown in Sabouraud (oxoid) liquid media until in the log stage of growth (cells were harvested

after 4 hours growth). They were then resuspended to an optical density of 0.87 at 650 nm, this is equivalent to 7.0 log $_{10}$ CFU / ml compared to 8.5 log $_{10}$ CFU / ml used for the other organisms. The illumination procedure was the same as used in previous experiments. Following illumination the yeast were plated out on Sabouraud dextrose agar (oxoid) and incubated for 24 hours at 37 °C, to assay for colony forming units.

Photobleaching 0.25mls of a 1mM solution of photosensitiser, 0.25mls of 10mM tryptophan was added to 25mls of 60% methanol, 40% potassium phosphate buffer (pH7.0). Experiments were also carried out in the absence of tryptophan where this was replaced by 0.25mls of the 60% methanol, 40% potassium phosphate buffer (pH7.0).

The mixture was illuminated as in the cell inactivation experiments above (1.3mW/cm^2) for 60 minutes, samples were taken every 15 minutes and spectra recorded on a UV-Visible spectrophotometer between 500nm and 700nm. For high light dose, illumination was at 9mW/cm^2 for 60 minutes.

Results

Antibacterial properties of phenothiazinium derivatives

Figure 4 shows log change in Colony Forming Units (CFU)/ml of *E.coli* incubated for 30 minutes with 10μM phenothiazinium compound and illuminated for 60 minutes at 1.3mW/cm$^{2.}$ Data were recorded of cell survival following a 60 minute illumination by a halogen lamp. It may be seen that there is substantial bacterial inactivation with the trend in the group being a decrease from methylene blue to ethylene blue, followed by an increase of almost 1000 fold up to the tetra-n-butyl phenothiazinium derivative. The longer chain phenothiazinium compounds then show reduced bacterial cell kill ability such that the tetra-n-hexyl derivative is almost inactive. The tetra-n-butyl phenothiazine led to the largest change in colony forming units per ml of 5.1 log$_{10}$ equivalent to a percentage cell survival of 0.001%. The

lowest change of 0.19 $\log_{10}$ CFU was using the tetra-n-hexyl derivative which is a cell survival of 65.3%. There was no cell inactivation with a light only control.

Figure 5 shows the log change in CFU/ml of *E.coli* incubated for 30 minutes with different concentrations of tetra-nbutyl phenothiazinium derivative and illuminated for 15 minutes at 1.3mW/cm$^{-2.}$ 10μM was the most effective concentration tested for bacterial inactivation using tetra-n-butyl phenothiazinium derivative. The log change in CFU/ml with this concentration was 4.59 $\log_{10}$ units. Cell kill effects were achieved with all of the concentrations tested but were reduced at the lower drug doses. At 50μM there is a log change of 2.15 units which is reduced compared to 10μM. This could be due to photosensitiser aggregation and therefore lower drug doses to the cell.

Many antibiotics are only poorly effective against non-growing or stationary bacteria and it is important to assess the ability of the phenothiazinium compounds to inactivate stationary phase bacteria. During the stationary period the cell has a thicker peptidoglyan cell wall and differences in protein metabolism and therefore might be less susceptable to the photodynamic effect. Figure 6 shows the log change in CFU/ml of *E.coli* in the stationary phase of growth following incubation for 30 minutes with 10μM phenothiazinium compounds and illuminated for 60 minutes at 1.3mW/cm^2. It may be seen that the effectiveness of the tetra-n-propyl and tetra-n-butyl derivatives is only slightly reduced against stationary phase bacteria, with again the tetra-n-butyl derivative being the most effective.

Inactivation of bacteria may be more challenging in a therapeutic environment, because the sensitiser may bind preferentially to extracellular proteins rather than the bacterial lipopolysaccharide membrane. This was tested by resuspending the bacteria in nutrient medium containing many factors which might compete with bacterial cells for photosensitiser binding. Figure 7 shows the log change in CFU/ml of *E.coli* resuspended in nutrient medium, from which it may be seen that there is little

reduction in the level of cell kill. Again, the tetra-n-butyl phenothiazinium derivative has the highest antibacterial activity.

Figure 8 shows the log change in CFU/ml of *E.coli* following incubation with 10μM phenothiazinium compound for 30 minutes and illumination with laser light (665nm) for only 4 minutes at 0.1W. Again, the same pattern of activity among the phenothiazine derivatives is seen, showing that the effects are present with coherent laser light. The potential advantages of a laser source are increased accuracy of light doses and shorter illumination times.

Further studies with laser light showed that a log change of 5.69 CFU/ml can be achieved with a 14 minute illumination at 0.1W with the tetra-n-butyl phenothiazinium derivative and that after a 20 minute illumination there is a log change of almost 8.5 units, though the number of CFU are too small to make this figure statistically reliable

Uptake of the photosensitisers into bacterial cells is clearly important in determining photo-activity. Figure 9 shows uptake of 10μM phenothiazinium compounds into *E.coli* cells following a 30 minute incubation. Cells were washed twice in 0.1M pH7.0 potassium phosphate buffer to remove extra-cellular or loosely bound sensitiser. It may be seen that uptake of the phenothiazinium compounds is somewhat correlated with phototoxicity in that the tetra-n-butyl derivative is taken up the most by the bacterial cells. However, the correlation between uptake and photoactivity is far from exact. For example, the ratio between the photoactivity and the uptake for the tetra-n-butyl derivative is far greater than that for the tetra-n-hexyl derivative. These ratios would be expected to be the same for all of the derivatives if the photoactivity could be explained only on the basis of uptake. It is therefore clear that the extremely high activities of the tetra-n-butyl and tetra-n-propyl derivatives must be due to some additional factors, as yet unknown.

Data not shown have proved that the tetra-n-butyl derivative is taken up very quickly into the *E.coli*; there are no differences in uptake between incubation times of 5 minutes and 30 minutes. However, in the presence of nutrient medium, the uptake was found to be somewhat slower and reduced in extent.

Figure 10 shows the log change in CFU/ml of *E.coli* cells incubated with 10μM tetra –n-butyl phenothiazinium derivative and then washed twice with 0.1M pH7 potassium phosphate buffer to remove any extra-cellular or loosely bound photosensitiser, which may have an effect on the phototoxicity. Illumination used laser light (665 nm) at 0.1 W for 4 minutes. The results show there is little difference between the log change in CFU/ml of washed and unwashed cells, indicating that it is the tightly bound photosensitiser which is causing cell death. At present, the precise location of the photosensitiser within the bacterial cell is not known but the photodynamic action is effective and non -recovering.

Data for the effect of the different phenothiazinium compounds on the growth of an *E.coli* culture in the dark as compared to a control are shown in Figure 11. Incubation was carried out in the dark at 37°C for 6 hours and measurements were based on apparent turbidity at 550nm as described earlier. All the cultures containing phenothiazinium compounds show reduced growth as compared to the control with the tetra-n-butyl phenothiazinium derivative showing the greatest inhibition in the number of cells in the bacterial suspension. It should be emphasised that this dark inhibition is many orders of magnitude less than that observed for cell inactivation in the light.

Further work was carried out with the tetra-n-butyl derivative alone to determine the effect of photosensitiser plus light on growth of bacteria. These data, shown in Figure 12 show clearly that for the growing bacteria with addition of photosensitiser after 3 hours, there is continuing growth in the dark, but complete elimination of growth in the light. The data again illustrate the very powerful photobacteriocidal effect of this photosensitiser.

Figure 13 shows percentage cell survival of *Pseudomonas aeruginosa* following incubation with 10μM tetra-n-butyl phenothiazinium derivative. Illumination was with laser light (665nm) at 0.1 W. *P aeruginosa* is a Gram negative organism which is commonly associated with a number of skin conditions including infections of ulcers and burn wounds. The figure shows that the tetra-n-butyl phenothiazinium derivative can photodynamically inactive this organism extremely efficiently. An illumination time of only 2 minutes with laser light (665nm) at 0.1 W leads to a greater than 99% cell kill, while increase of the illumination time to 10 minutes gives almost 4 logs of cell kill. Control studies showed that there is no reduction in cell number caused by the illumination alone in the absence of photosensitiser with 10μM tetra-n-butyl phenothiazinium derivative .

Figure 14 shows percentage cell survival of *Staphylococcus aureus* following incubation with 10μM tetra-nbutyl phenothiazinium. Illumination was with laser light (665 nm) at 0.1 W. *S.aureus* is a Gram positive organism which differs from Gram negative organisms in that it has a thick outer peptidoglycan layer and no external lipopolysaccharide. The bacterial structure is the same as in MRSA (Methicillin resistant *S.aureus)* which is resistant to almost all commonly used antibiotics. The data show that after only a 1 minute illumination almost 99% of the bacteria are inactivated and that after 10 minutes there is almost 5 logs of cell kill, illustrating the very high photoactivity of the tetra-n-butyl derivative against this Gram positive organism.

It is important to determine if the photosensitiser would also be active against the antibiotic resistant form, MRSA, as this would have major health and industrial applications. Figure 15 shows percentage cell survival of MRSA following illumination with 665 nm laser light at 0.1W and incubation with 10μM tetra-n-butyl phenothiazinium derivative. The data clearly show that this photosensitiser is indeed highly photoactive in killing MRSA.

Anti-fungal properties of phenothiazinium derivatives

In order to test the ability of the tetrabutyl derivative to kill fungal cells in the light, the photosensitiser was incubated with cells of *Candida albicans* and the culture was subjected to laser light as described above. The results are shown in Figure 16, in which it is clear that this eukaryotic organism is also readily destroyed. This photosensitiser is therefore also highly photoactive against this fungal organism which is responsible for many common infections e.g.thrush.

Selectivity for bacterial cells versus mammalian tissues

It is clearly important for therapeutic purposes that there is minimal damage to host tissues while microorganisms are being destroyed. This was tested by applying a solution of the tetra-n-butyl phenothiazinium derivative to the ears of experimental mice and illuminating, under conditions in which the total dose was almost 20 times that needed for bacterial or fungal elimination. The possible effects on the host tissue were assessed by measuring any increase in ear thickness. This is a standard model for detecting photodynamic reactions in the skin. Figure 17 shows the data obtained, compared with results from intravenous administration of PHP, a drug equivalent to Photofrin which is known to cause prolonged skin reaction. It is clear from Figure 17 that, while the reaction from PHP is very strong, as expected, there is little or no reaction from the tetra-n-butyl phenothiazinium derivative, suggesting that mammalian tissues would not be damaged during antimicrobial treatment.

Photobleaching

Photobleaching removes detectable colour from the photosensitiser, rendering it inactive and is the result of the its instability to light and reduction or oxidation. Such photobleaching may have advantages or disadvantages depending on the potential application. For example, photobleaching is undesirable in the coating of lines and catheters. Two sets of experiments were carried out; one at a high light dose ($9.0mW/cm^2$) and one at a low light dose ($1.3mW/cm^2$) with and without tryptophan as described above. Absorption spectra at low light dose, with and without tryptophan, showed no changes for any of the phenothiazinium compounds demonstrating they are stable at this dose. At the high light dose, spectral changes

were observed for the methylene blue, indicating photobleaching. The maximum absorbance decreased and the wavelength peak shifted over the one hour illumination. These changes occurred to the same extent with and without tryptophan. However, none of the other phenothiazinium compounds showed this degradation and remained stable to photobleaching at the high light dose.

Phenothioazinium compounds of structure I suitable for inclusion in polymers or attachment to, or adsorption on, polymer surfaces

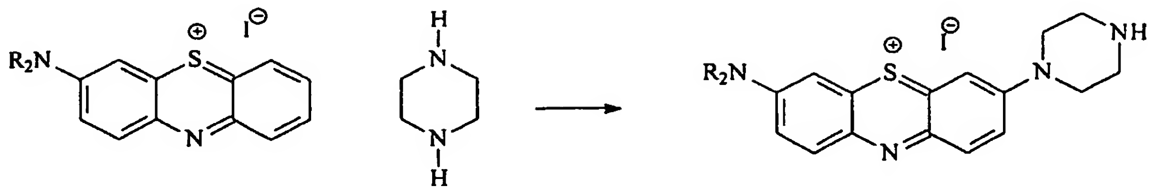
(a) <u>Inclusion within polymers</u>

Example

To a clear solution of cellulose triacetate (0.5 g) in dichloromethane (10 cm^3) was added sensitiser **(I, X = Y = n-Bu)** (0.01g) and the mixture was stirred until the sensitiser had dissolved completely. The solution was then cast on a glass plate and allowed to dry slowly, giving a clear blue film. The film showed typical singlet oxygen generating properties on exposure to light. Thus an aerated red solution of tetraphenylcyclopentadienone (a characteristic singlet oxygen detector) in toluene containing the film was rapidly bleached on exposure to light from a 40 w tungsten filament lamp. An identical solution showed no bleaching when irradiated for the same period of time in the absence of the film.

(b) <u>Adsorption on polymers</u>

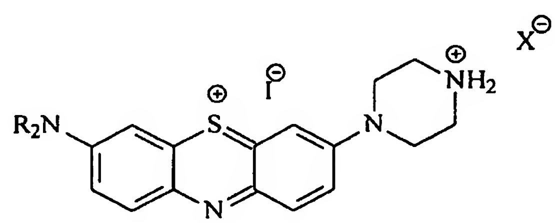
Phenothiazinium compound **Ia** and **Ib** were made according to the following reaction scheme and were isolated as dark blue solids. They were characterised by mass spectrometry.



Ia; R = n-Pr

Ib; R =n-Bu

Compounds (**Ia** and **Ib**) were extremely basic and readily protonated in dilute acids to give (**IIa**) and (**IIb**) respectively, which could be adsorbed strongly on polymeric surfaces, e.g. polyamides, polyacrylates, polyesters, polycarbonates, polyurethanes, and strongly resisted removal by water or solvents. Alternatively **Ia** or **Ib** could be adsorbed directly onto acidic surfaces to give their corresponding cationic salts directly.



IIa; R = n-Pr

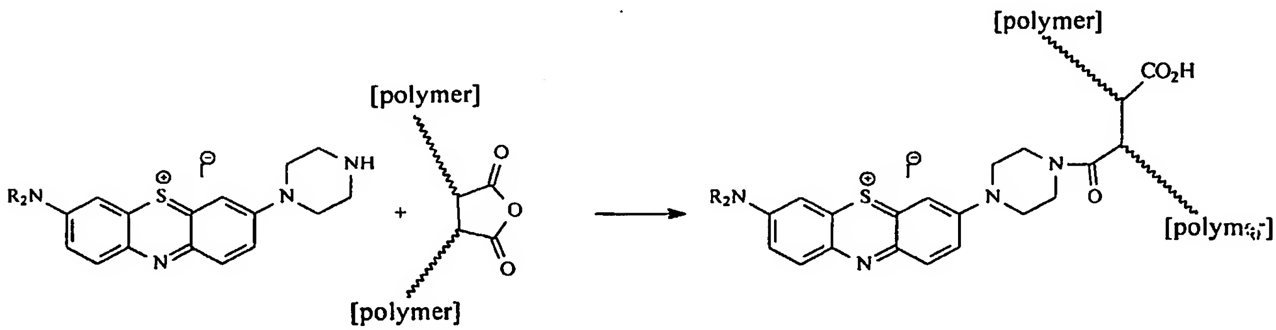
IIb; R = n-Bu

(c) <u>Covalent attachment of the phenothiazinium sensitisers to polymer substrates</u>

<u>Derivatives Ia and Ib</u>

Compounds **Ia** and **Ib** proved very reactive as nucleophiles in various substitution reactions that could provide a means of attaching the sensitiser unit covalently to polymers.

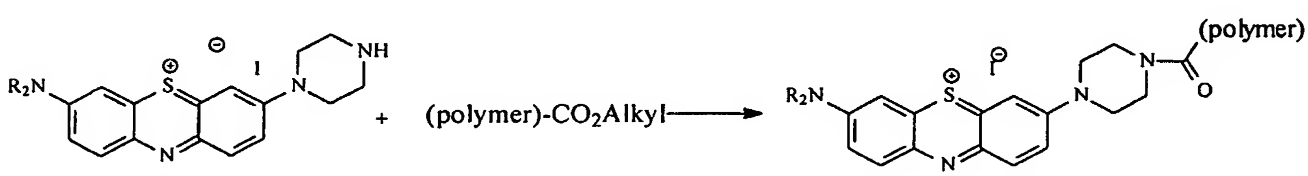
Thus reaction with anhydrides occurred, as exemplified by the following reaction:



Example:

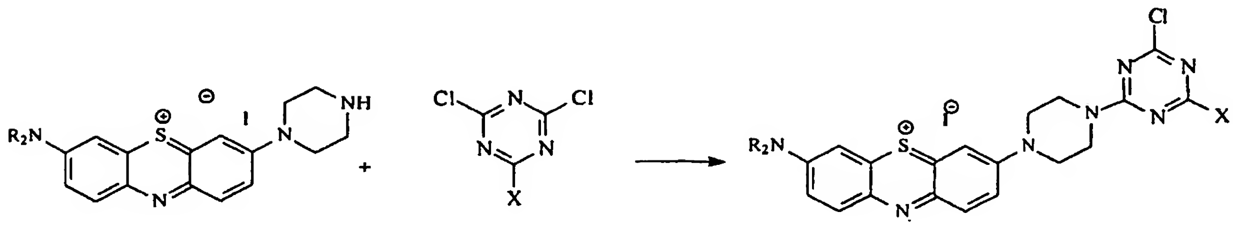
Polyethylene-*graft*-maleic anhydride (1.0 g) was dissolved in toluene (25 cm^3) with warming. The sensitiser **Ia** (0.20 g) was added and reaction mixture heated under reflux for 1 hour. The mixture was poured into methanol and the precipitate was filtered off, washed with methanol and dried, giving the sensitiser- bound copolymer as a dark blue powder (1.1 g). Covalent attachment of the sensitiser to the polymer was confirmed by dissolving the powder in dichloromethane and precipitating it by addition of methanol. No blue colour remained in the supernatant liquid.

A similar nucleophilic substitution reaction will occur with polymers containing ester



groups, i.e. :

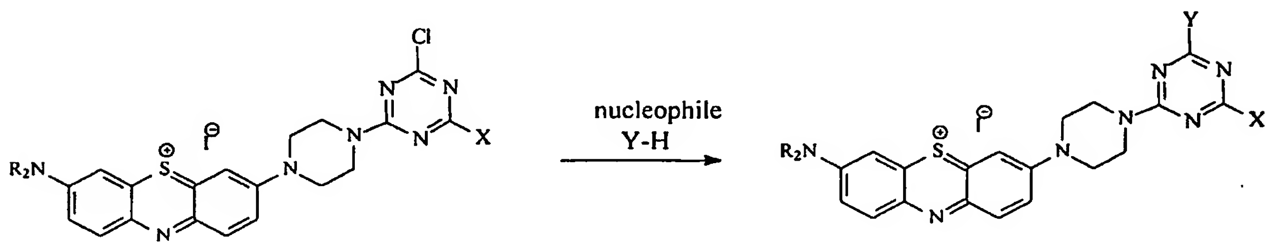
Phenothiazinium derivatives **Ia** and **Ib** are also very reactive towards chlorotriazine derivatives, and their linkage to polymers can be carried out by the following procedure:



Where X = -NH-(Polymer) in the case of polyamide polymers

X = -O-(Polymer) in the case of cellulosic polymers

Alternatively, the residual chlorine in the previous example can be replaced by other reactive groups, as in the following reaction:



Where X and Y = -NH-(polymer)

Or X and Y = -O-(polymer)

Or X may be an amine –NHR or –NRR', and Y = -NH-(polymer), or –O-(polymer)

These are not the only means of attaching the phenothiazinium derivatives to polymers, and other methods may by employed based on existing polymer-grafting chemistry known to those skilled in the art.

Example:

Sodium carbonate (0.20 g) and cyanuric chloride (0.30 g) were added to a solution of the sensitiser **Ia** (0.26 g) in dry acetone (170 cm^3) at room temperature, and the mixture was stirred for 15 minutes. A sheet of transparent cellulose film (2.2 g) was immersed in an aqueous solution of sodium hydroxide (1M; 500 cm^3) for 10 minutes and then washed free of sodium hydroxide. This was then introduced into the sensitiser solution and the stirred mixture heated at 50 °C for 15 minutes. Water (200

ml) was added and the mixture heated at 60°c for 30 minutes. The blue cellulose film was then removed and washed with water, and then heated in sodium carbonate solution (6%)to remove any unfixed dye. Covalent attachment to the cellulose was confirmed by heating the film in boiling sodium carbonate solution or boiling methanol, when no blue colour was removed. The film showed typical singlet oxygen generating properties on exposure to light, and when immersed in an air-saturated solution of tetraphenylcyclopentadienone in toluene and exposed to light from a 40 w tungsten filament lamp, the red dienone was bleached more rapidly than an identical solution containing no film.

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Wagner SJ, Skripchenko A, Robinette D, Foley JW, Cincotta L (1998). Factors affecting virus photoinactivation by a series of phenothiazine dyes. Photochemistry and Photobiology **67**, 343-349.

Table 2. Chemical properties of the phenothiazines and phototoxicity, dark toxicity, cellular uptake and subcellular localisation in RIF-1 murine fibrosarcoma cells.

R	Singlet oxygen generation[1]	PDT LD_{50} (μM)[2]	Ratio dark : PDT LD_{50}[2]	Uptake at PDT LD_{50} (nmol / mg protein)[3]	Initial localisation[4]	Relocalisation + light[4]	LogP
Methyl	47	54	3	> 4.6	Lysosomes	Nucleus	-1.0
Ethyl	42	3.9	27	2.9	Lysosomes	Lysosomes	+0.2
Propyl	40	0.42	19	0.9	Lysosomes	Mitochondria	+1.1
Butyl	41	1.1	7	3.1	Lysosomes	Mitochondria	+1.1
Pentyl	39	0.74	10	1.6	Lysosomes	Mitochondria	+1.7
Hexyl	35	2.1	4	1.6	Lysosomes	Mitochondria	+1.3

[1] % photo-oxidation of 1,3-diphenylisobenzofuran after 10 min red light illumination with 100mg/ml of the phenothiazine in 90% DMF : 10% water.

[2] Cells were incubated with the phenothiazine for 2h. For measurement of phototoxicity, cells were illuminated with 3J / cm^2 (10mW / cm^2) white light. Dark toxicity was measured in parallel. Cell survival was assessed after 24h using the MTT assay.

[3] Cells were incubated with the phenothiazine for 2h. Cells were solubilised in 2% SDS and the phenothiazine levels measured by fluorescence.

[4] Cells were incubated with the PDT LD_{50} concentration of the phenothiazine for 2h and fluorescence images captured before and during 10min illumination with 630nm light.

Table 3. Phototoxicity and dark toxicity of the phenothiazines in human tumour cell lines.

R	OE33[1]	SiHa[2]	HT1376[3]	HT29[4]
Methyl				
- PDT LD_{50} (μM)	43.5 ± 1.8	18.7 ± 1.0	37.9 ± 10.1	88.5 ± 6.1
- Ratio dark : PDT LD_{50}	2.0	1.0	1.6	1.5
Propyl				
- PDT LD_{50} (μM)	0.30 ± 0.09	0.075 ± 0.015	0.20 ± 0.13	0.22 ± 0.04
- Ratio dark : PDT LD_{50}	11	80	13	18
Butyl				
- PDT LD_{50} (μM)		0.28 ± 0.06		
- Ratio dark : PDT LD_{50}		18		
Pentyl				
- PDT LD_{50} (μM)		0.29 ± 0.06	0.75 ± 0.22	1.49 ± 0.41
- Ratio dark : PDT LD_{50}		11	6	4

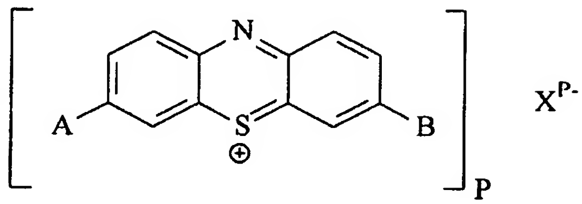
[1] oesophageal adenocarcinoma
[2] cervical squamous cell carcinoma
[3] bladder transitional cell carcinoma
[4] colon adenocarcinoma

Cells were incubated with the phenothiazine for 2h. For measurement of phototoxicity, cells were illuminated with 3J / cm^2 (10mW / cm^2) 665nm light. Dark toxicity was measured in parallel. Cell survival was assessed after 48h using the sulforhodamine B (SRB) assay.

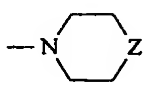
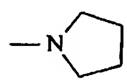
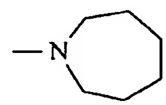
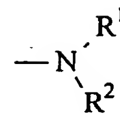
CLAIMS

1. A phenothiazinium compound of Formula (I):

(I)



wherein A and B are each independently

   or 

in which Z is CH_2, O, S, SO_2, NH, NCH_3, NC_2H_5, NCH_2CH_2OH, or $NCOCH_3$ and R^1 and R^2 are each independently linear or branched $C_nH_{2n}Y$, where n is 1-6, Y is H, F, Cl, Br, I, OH, OCH_3, OC_2H_5, OC_3H_7, CN or $OCOCH_3$,

and where X^{P-} is a counteranion and P is 1, 2 or 3

but not including a compound where A and B are both either $-N(CH_3)_2$ or $-N(CH_2CH_3)_2$.

2. A compound according to claim 1 wherein the counteranion is selected from any of Cl^-, Br^-, I^-, F^-, NO_3^-, HSO_4^-, $CH_3CO_2^-$, or a dianion, namely, SO_4^{2-} or HPO_4^{2-}, or a trianion namely PO_4^{3-}.

3. A compound according to claim 1 wherein A and B are the same and both R^1 and R^2 are n-propyl, n-butyl or n-pentyl.

4. A compound according to any of the preceding claims for use against microorganisms.

5. A compound according to claim 4 for use against bacteria.

6. A compound according to claim 4 or claim 5 for use against antibiotic resistant bacteria.

7. A compound according to any of claims 1 to 3 for use as a PDT agent or a photodiagnostic agent.

8. A compound according to any of claims 1 to 3 for use as a medicament.

9. A compound according to any of claims 1 to 3 for use as an anti-microbial treatment for skin and other local infections, for sterilisation of burn wounds and other lesions, and for the treatment of dental bacterial disease.

10. A compound according to any of claims 1 to 3 for use in the treatment of pre-cancerous conditions, cancer, ophthalmological disease including macular degeneration, vascular problems such as cardiovascular disease, arteriosclerosis and restenosis and autoimmune diseases such as rheumatoid arthritis, skin diseases such as psoriasis, acne and excema and other benign conditions such as endometriosis and menorrhagia.

11. A compound according to any of claims 1 to 3 for use as a photoactivated antimicrobial agent for sterilisation of surfaces and fluids.

12. A compound according to any of claims 1 to 3 for use in photochemical internalisation.

13. A compound according to any of claims 1 to 3 for use in photodetection and/or photodiagnosis.

14. A conjugate or composite formed between a compound of any of the preceding claims and a polymer.

15. A conjugate or composite of claim 14 wherein the polymer includes anhydride and/or ester groups.

16. A compound formed by the reaction between a compound of any of claims 1 to 13 and a chlorotriazine derivative.

17. A compound according to claim 16 wherein the chlorotriazine derivative is a polymer having chlorotriazine groups attached thereto.

18. A composition comprising a compound, conjugate or composite of any of claims 1 to 17 together with a diluent or excipient.

19. A method of treating pre-cancerous conditions, cancer, ophthalmological disease including macular degeneration, vascular problems such as cardiovascular disease, arteriosclerosis and restenosis and autoimmune diseases such as rheumatoid arthritis, skin diseases such as psoriasis, acne and excema and other benign conditions such as endometriosis and menorrhagia, the method comprising administering to a subject a therapeutically effective amount of a compound of any of claims 1 to 3 and exposing said subject to light to render active said compound.

20. A method according to claim 19 wherein said compound administered is as defined in claim 3 where R^1 and R^2 are n-propyl and said light exposure is given up to 10 minutes from the point of drug administration.

21. A method according to claim 20 wherein light exposure is given within 1 minute of drug administration.

22. A method according to claim 20 wherein light exposure is give at the point of drug administration.

23. A method according to claim 19 wherein the compound administered is as defined in claim 4 where R^1 and R^2 are n-pentyl and said light exposure is give up to one hour from the point of drug administration.

24. A method of treatment of microbial infections, burn wounds and other lesions and of dental bacterial disease, the method comprising applying to the area to be treated a therapeutically effective amount of a compound of any of claims 1 to 3 and exposing said area to light to render active said compound.

25. A method according to claim 24 wherein the compound administered is as defined in claim 5 where R^1 and R^2 are n-butyl.

26. A method of sterilising a surface or a fluid comprising applying the compound of any of claims 1 to 3 to said surface or fluid and activating said compound by means of light.

27. An article having at least one surface to which is attached a compound, conugate or composite of any of claims 1 to 3.

28. An article according to claim 27 wherein attachment is by covalent bonds or by intermolecular interactions.

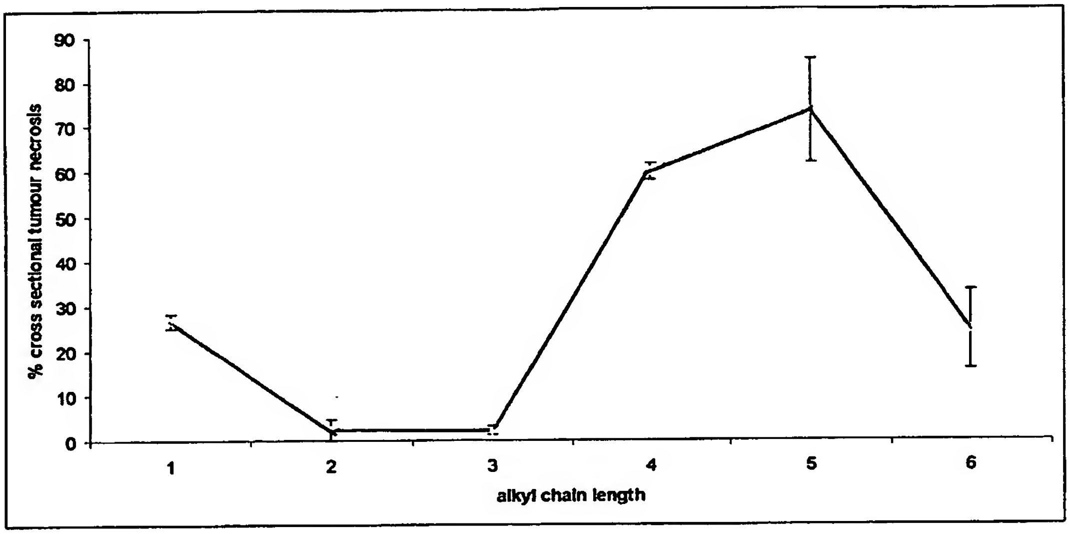
29. An article according to claim 27 or claim 28 which is a medical device.

30. An article according to claim 27 or claim 28 which is for use in the food industry.

Figure 1

Symmetrical phenothiazinium salts: *in vivo* activity at 1h

Figure shows % cross sectional tumour necrosis at 72h post-PDT. All drugs were administered i.v. at a dose of 16.7μmol/kg. At 1h post drug administration light (60J/cm^2, 50mW/cm^2) was administered superficially.



Symmetrical phenothiazinium salt	Alkyl chain length	Vehicle	Wavelenght (nm ± 15)	%area	s.e.m
Methyl	1	Phys. saline	685	26.70	1.60
Ethyl	2	Phys. saline	630	2.38	2.38
Propyl	3	2%DMSO/H2O	630	2.27	0.99
Butyl	4	2%DMSO/H2O	660	59.74	1.78
Pentyl	5	2%DMSO/H2O	685	73.31	11.57
Hexyl	6	2%DMSO/H2O	660	24.71	8.74

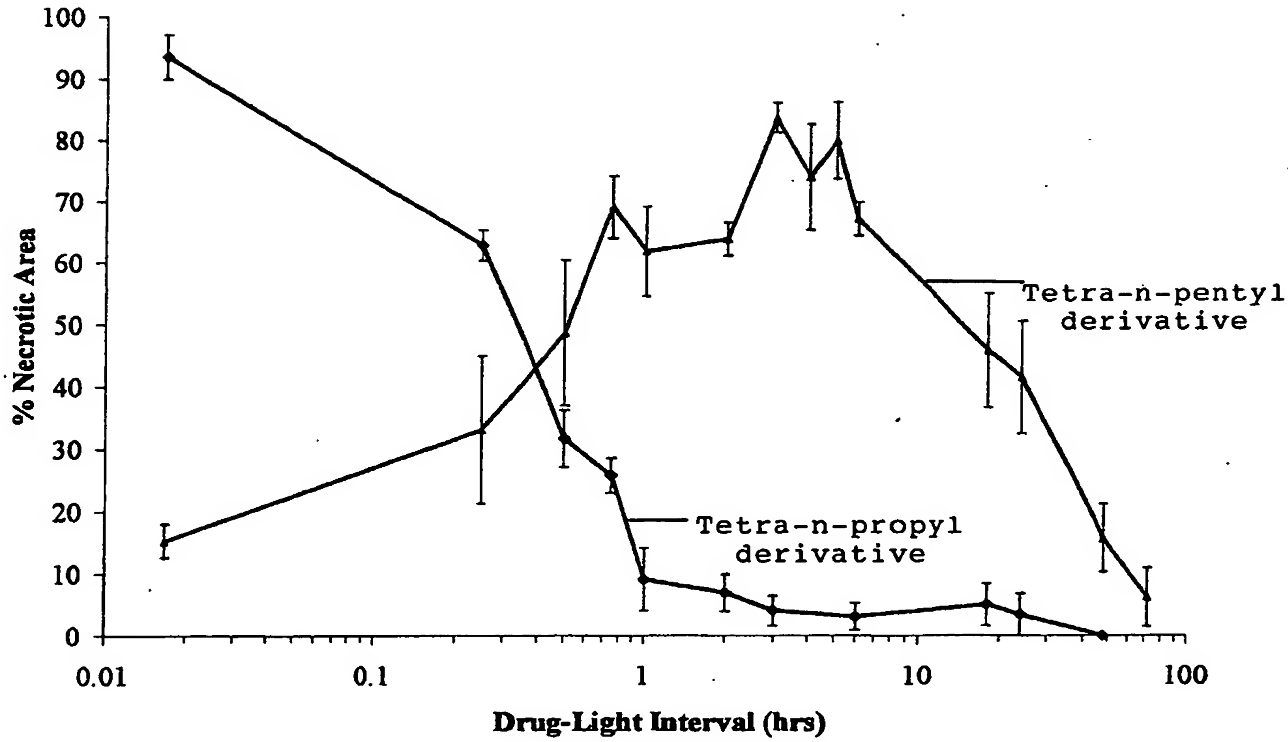


Figure 2: Area of tumour necrosis (expressed as a % total section area) 72 hrs after PDT with tetra-n-propyl and tetra-n-pentyl derivative (16.7μmolkg^{-1}, 660nm light @ 50mWcm^{-2}, 60Jcm^{-2}). Data points represent mean + SEM (n=6, each reading measured in triplicate).

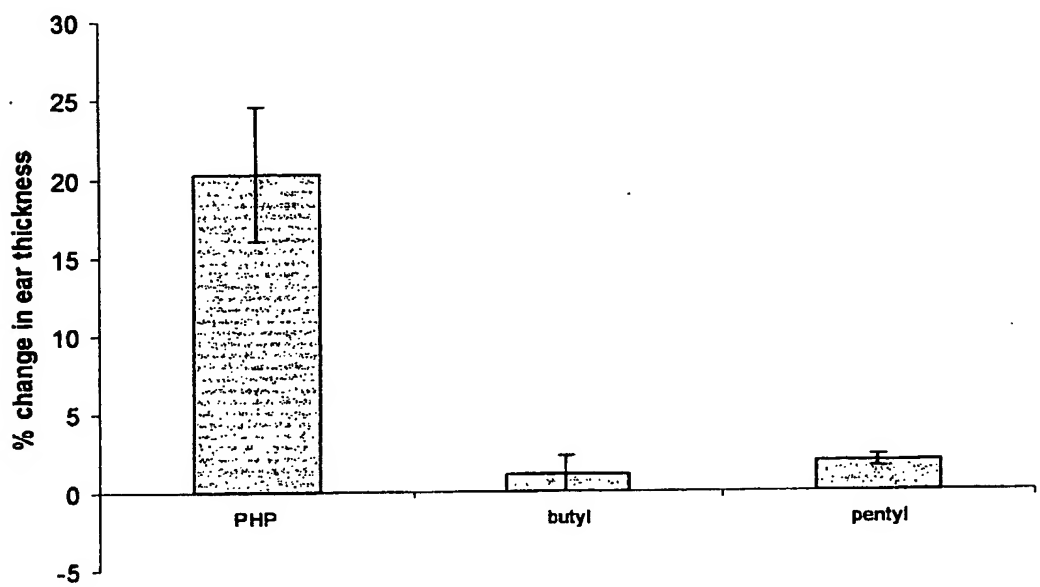
Figure 3

Skin photosensitivity- murine ear swelling response

CBA/Gy mice were injected with sensitiser at 16.7 μmol/kg. At 24h post drug injection ears were exposed to broad band white light from a xenon arc lamp (25J/cm^2, 30mW/cm^2). % Change in ear thickness was measured as :

(ear thickness at 24h post illumination - ear thickness pre-illumination) / ear thickness pre-illumination x 100

Increased % change in ear thickness measures increased skin photosensitivity.



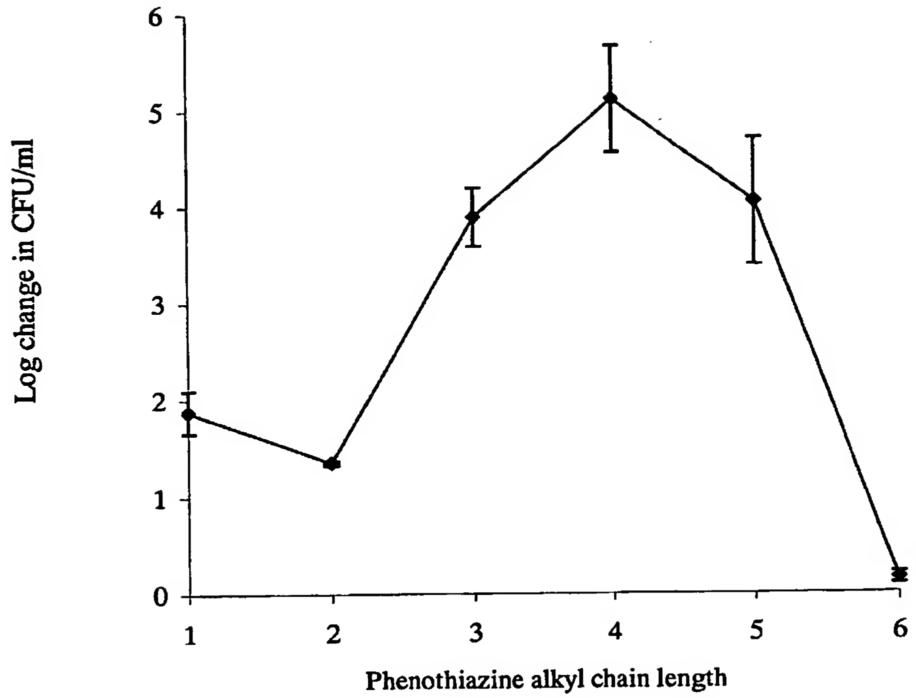


Figure 4 Log change in CFU/ml of *E.coli* incubated for 30 minutes with 10μM phenothiazine and illuminated for 60 minutes at 1.3mW/cm^{-2}

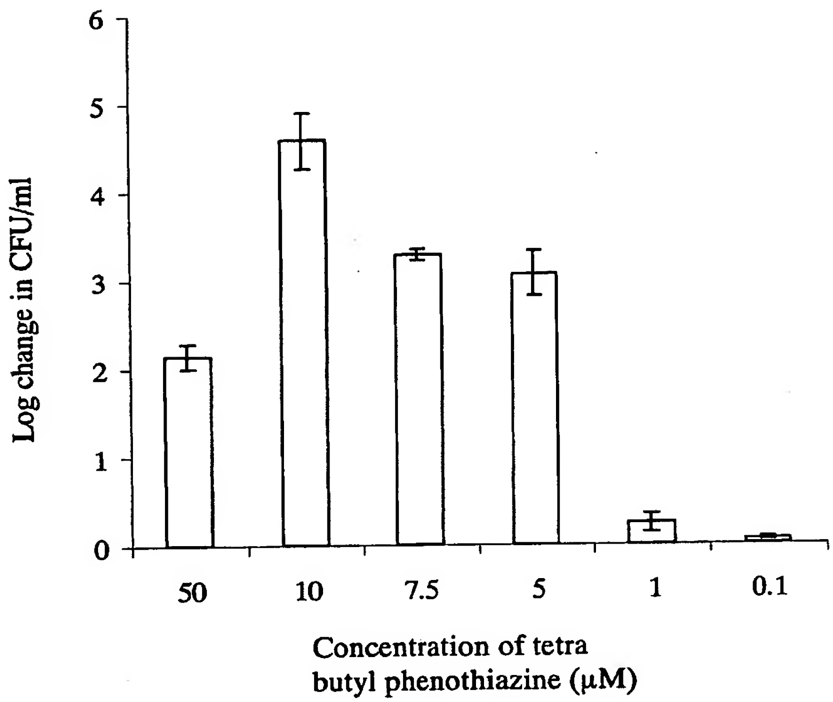


Figure 5 Log change in CFU/ml of *E.coli* incubated for 30 minutes with different concentrations of tetra butyl phenothiazine and illuminated for 15 minutes at 1.3mW/cm^{-2}

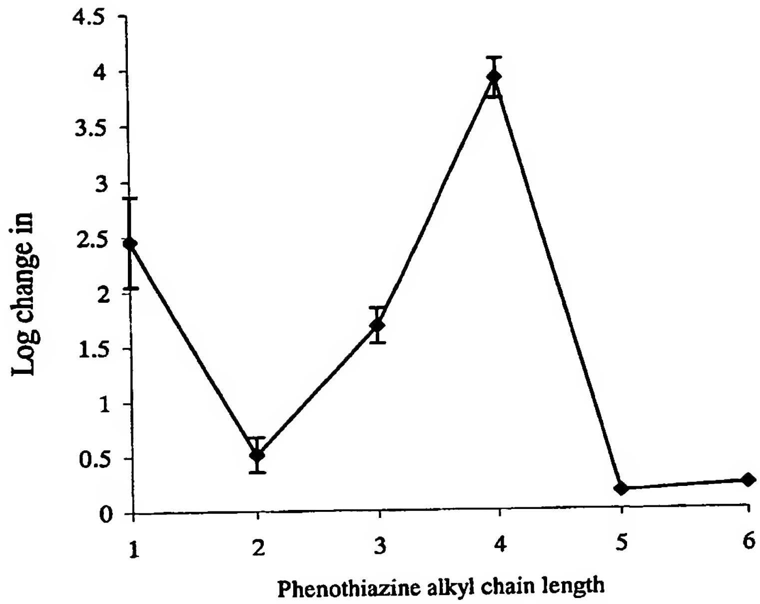


Figure 6 Log change in CFU/ml of *E.coli* in the stationary phase of growth following incubation for 30 minutes with 10μM phenothiazine and illuminated for 60 minutes at 1.3mWcm^{-2}

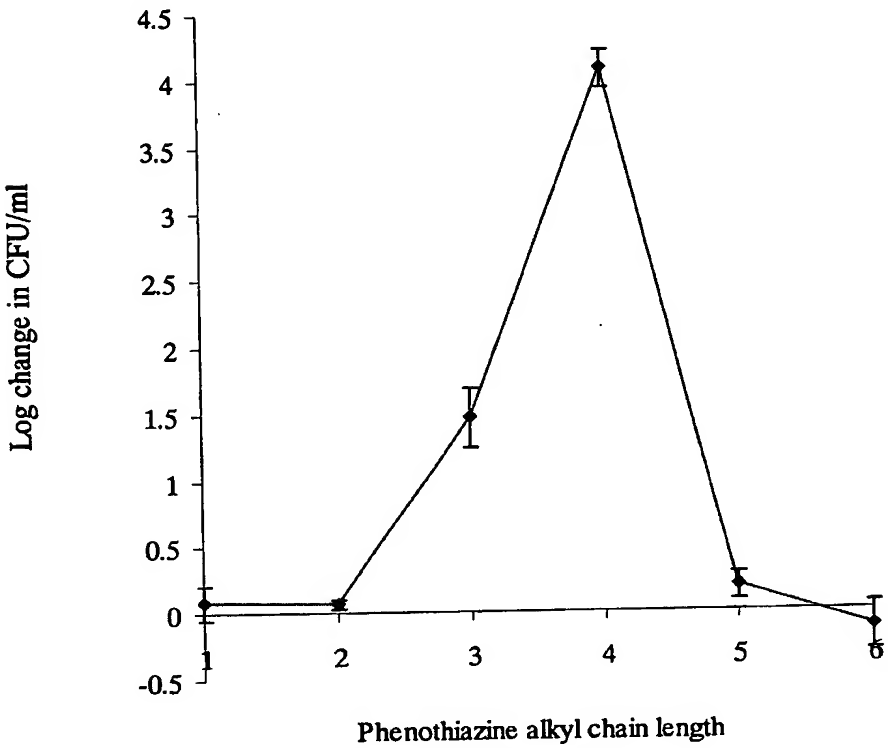


Figure 7 Log change in CFU/ml of *E.coli* resuspended in nutrient media. Cell were incubated for 30 minutes with 10μM phenothiazine and illuminated for 60 minutes at 1.3mW/cm^{-2}

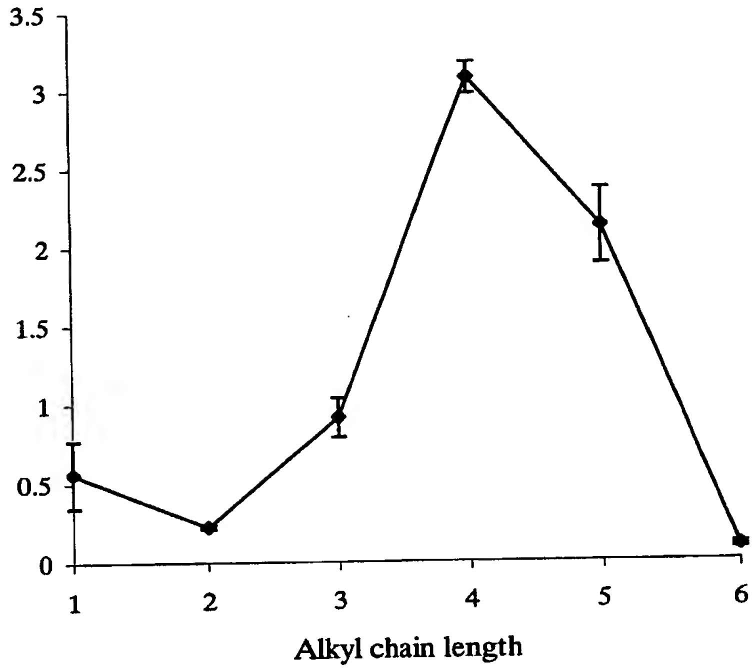


Figure 8 Log change in CFU/ml of *E.coli* following incubation with 10µM phenothiazine for 30 minutes. Illumination was with laser light (664nm) for 4 minutes at 0.1W

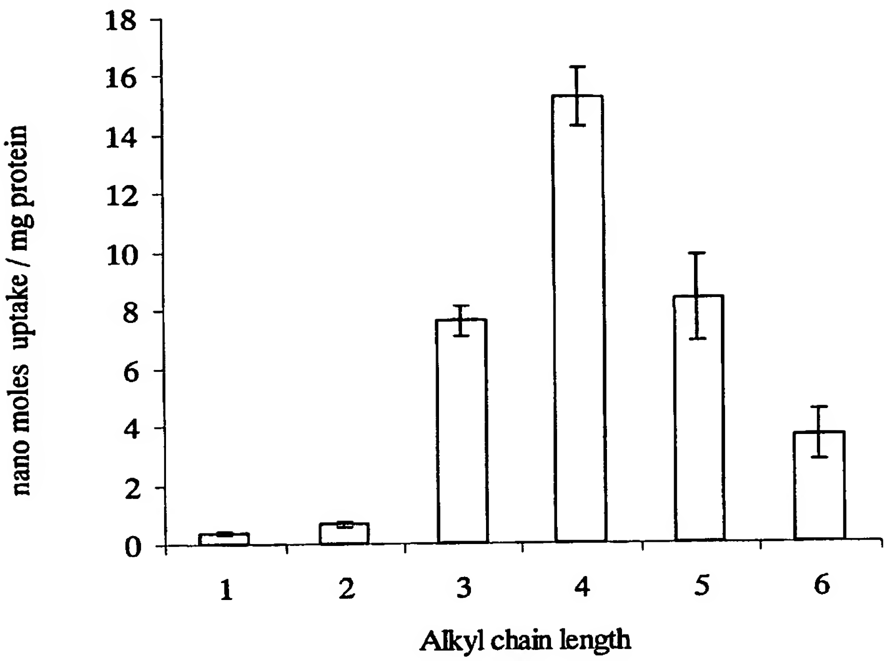


Figure 9 Uptake of 10μM phenothiazine into *E.coli* cells following a 30 minute incubation. Cells were washed twice in 0.1M pH7.0 potassium phosphate buffer to remove extra-cellular or loosely bound sensitiser.

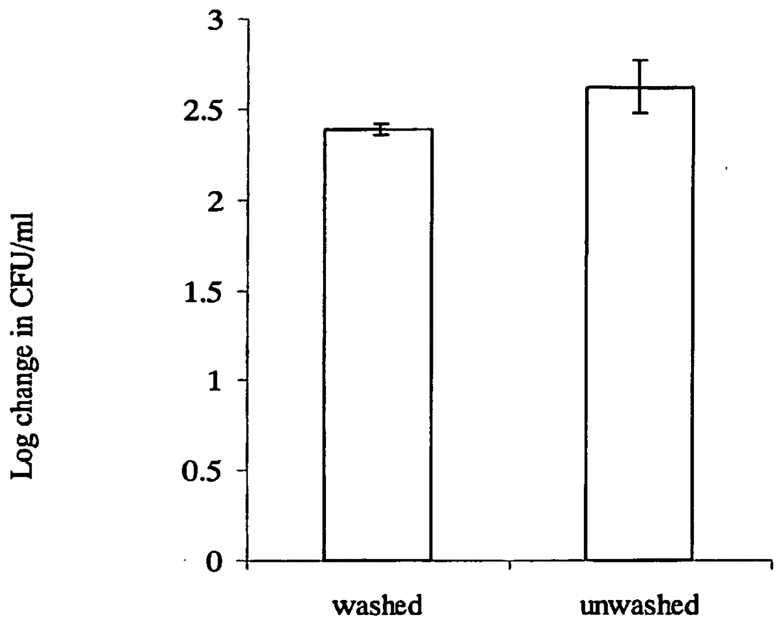


Figure 10 Log change in CFU/ml of *E.coli* cells incubated with 10μM tetra butyl phenothiazine. Cells were washed twice with 0.1M pH7 potassium phosphate buffer. Illumination used laser light (664nm) at 0.1W for 4 minutes.

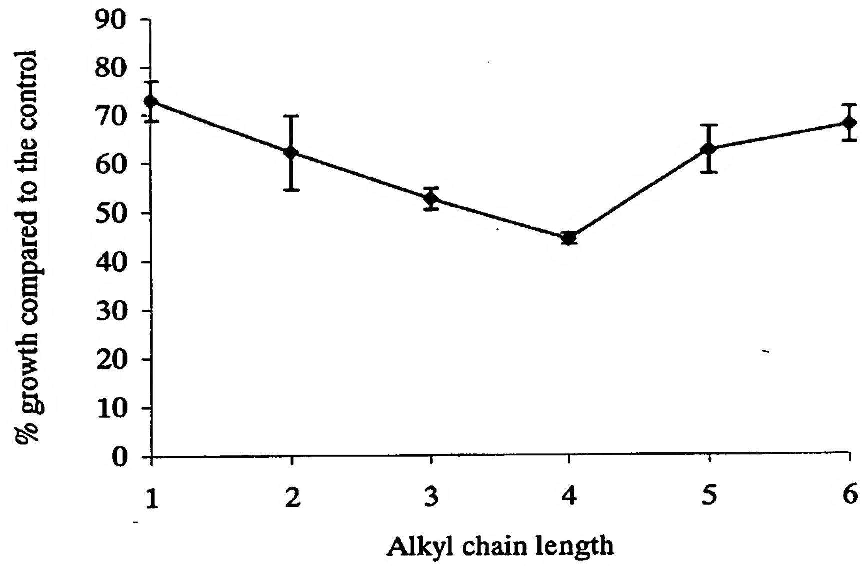


Figure 11 Percentage growth of a culture of an *E.coli* culture as compared to a control when 10µM phenothiazine was included in the growth media. Incubation was carried out in the dark at 37°C for 6 hours. Measurements based on apparent turbidity at 550nm.

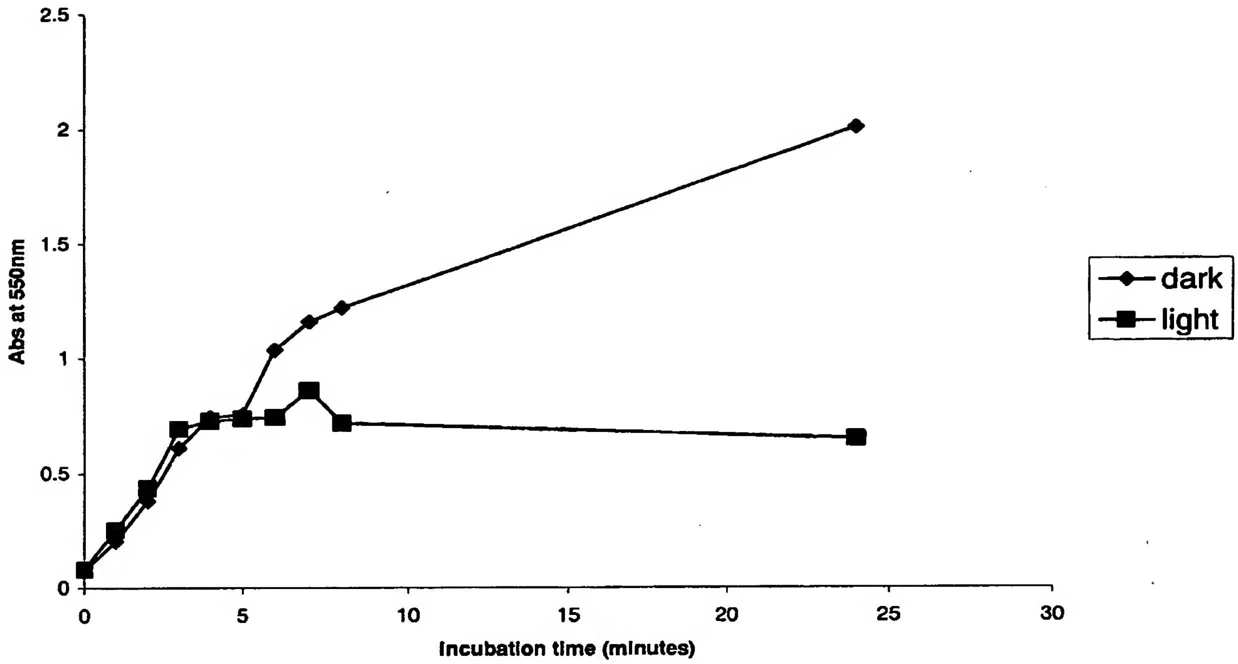


Figure 12 Change in absorbance of an *E.coli* culture grown in the presence of 10μM tetra butyl phenothiazine in the light and in the dark

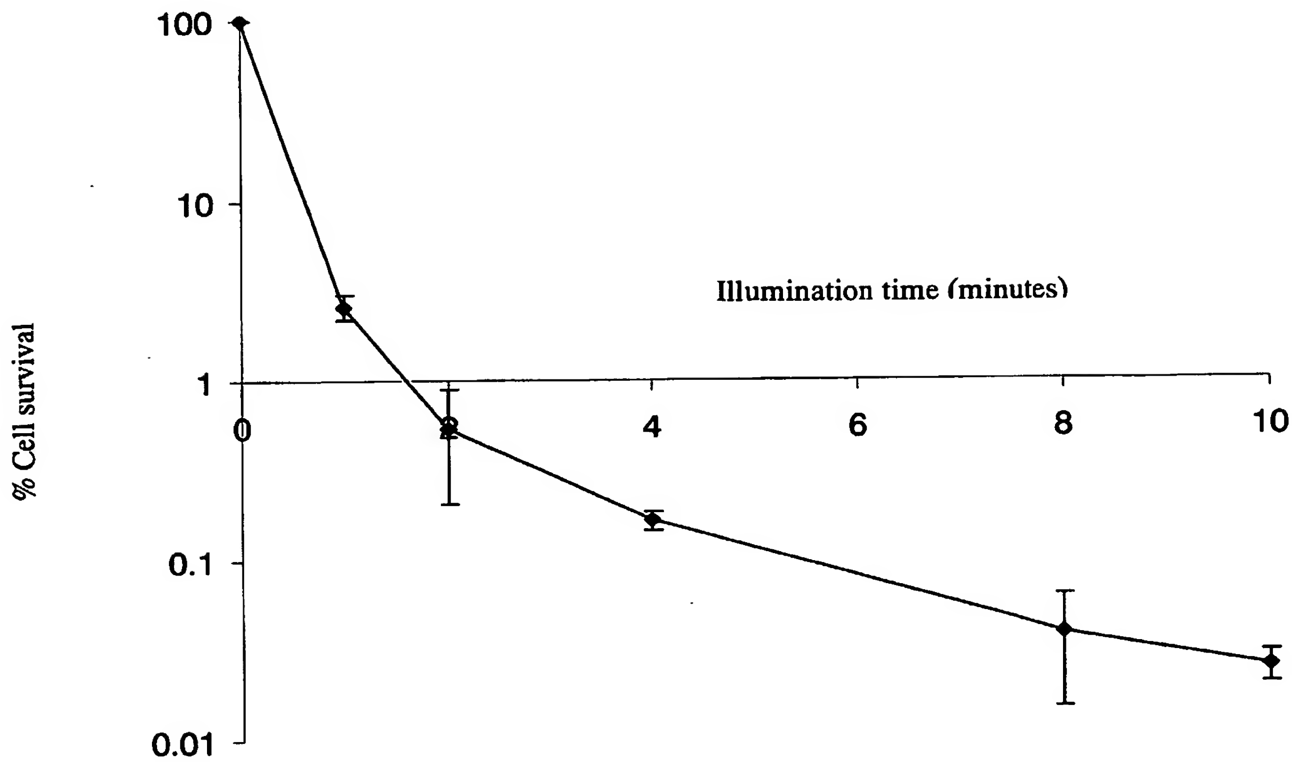


Figure 13 Percentage cell survival of *P.aeruginosa* following incubation with 10µM tetra butyl phenothiazine. Illumination was with laser light (664nm) at 0.1 W.

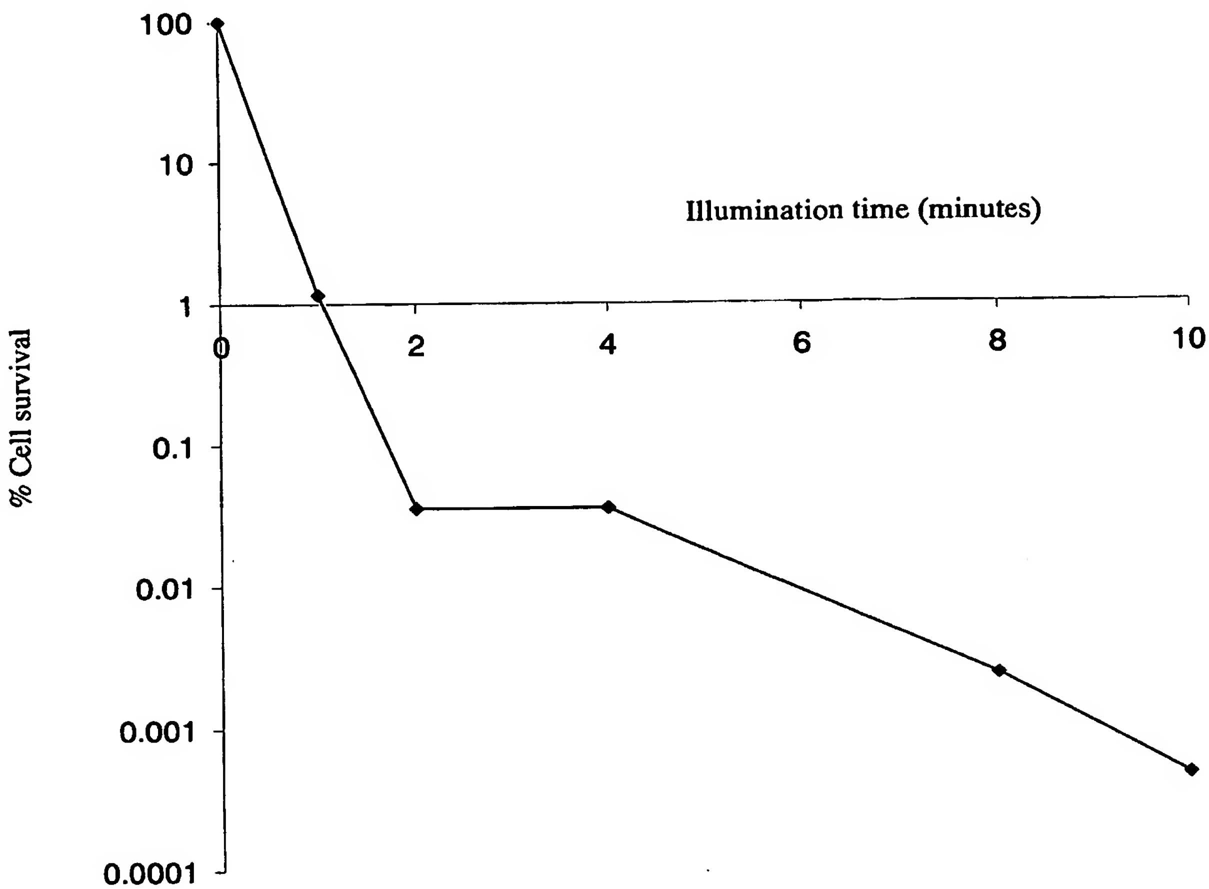


Figure 14 Percentage cell survival of *S.aureus* following incubation with 10µM tetra butyl phenothiazine. Illumination was with laser light (664nm) at 0.1 W.

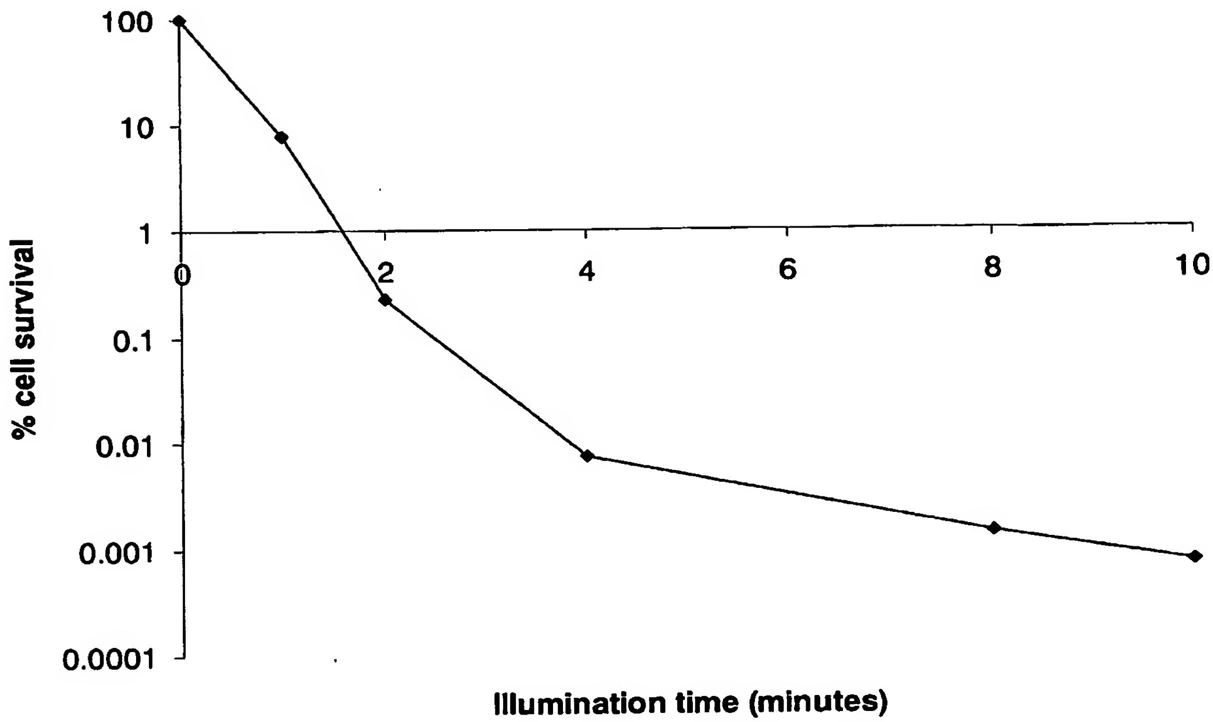


Figure 15 Percentage cell survival of MRSA following incubation with 10µM tetra butyl phenothiazine. Illumination was with laser light (664nm) at 0.1W

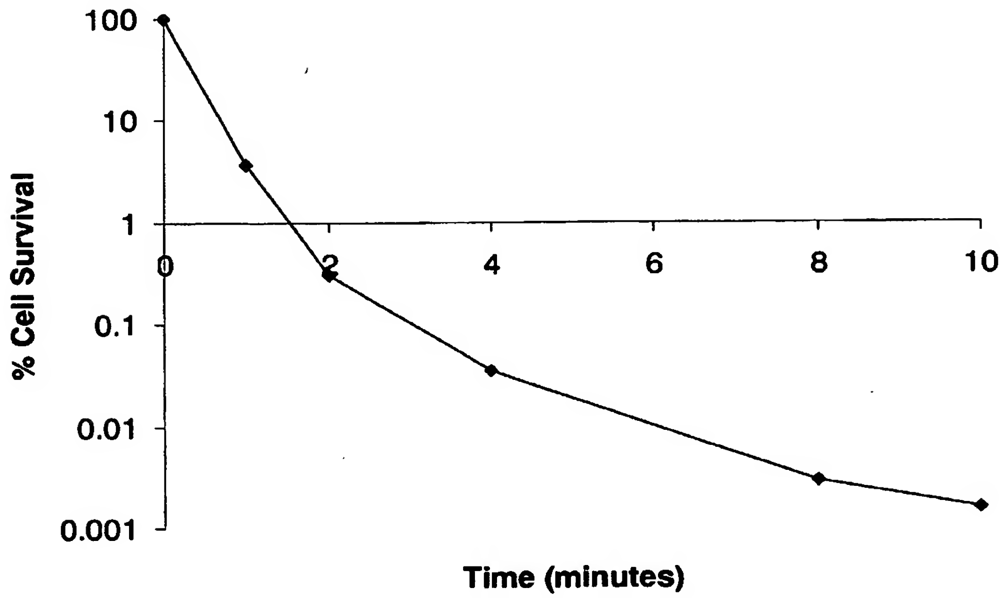
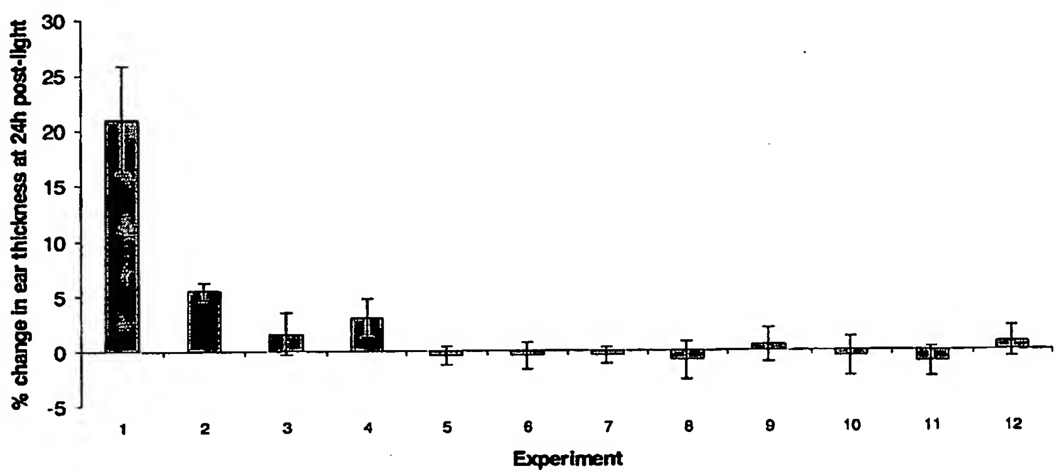


Figure 16 Percentage cell survival of *C.albicans* following incubation with 10µM tetra butyl phenothiazine. Illumination was with laser light (664nm) at 0.1W

Figure 17
Protocol:
Drug applied topically, dose = 5.79 µg (20µl at 0.5mM)
Applied light dose = 25J/cm^2 (30mW/cm^2 for 831sec)

1	PHP (i.v.) 8.35 µmol/kg 24h drug to light interval (solar simulator)
2	PHP(i.v.) 8.35 µmol/kg 2wk drug to light interval (solar simulator)
3	Butyl phenothiazinium drug only for 30min
4	24h ROOM LIGHT + Butyl phenothiazinium
5	24h ROOM LIGHT
6	24h DARK + Butyl phenothiazinium
7	24h DARK
8	Butyl phenothiazinium 30min drug to light interval (660±15nm)
9	Butyl phenothiazinium 30min drug to light interval (solar simulator)
10	Butyl phenothiazinium 24h drug to light interval (solar simulator)
11	Butyl phenothiazinium 7 days drug to light interval (solar simulator)
12	Butyl phenothiazinium 14 days drug to light interval (solar simulator)



INTERNATIONAL SEARCH REPORT

PCT/GB 02/02278

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07D279/18 A61K31/5415 A61P35/00 A61P31/04

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07D A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, BEILSTEIN Data, CHEM ABS Data, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	MELLISH, KIRSTE J. ET AL: "In vitro photodynamic activity of a series of methylene blue analogues" PHOTOCHEMISTRY AND PHOTOBIOLOGY (2002), 75(4), 392-397 , XP001094693 the whole document --- -/--	1-30

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 August 2002	26/08/2002

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Kollmannsberger, M

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	K. J. MELLISH, R. D. COX, D. I. VERNON, J. GRIFFITHS, S. B. BROWN: "In Vitro Activity of a Series of Novel Methylene Blue Analogues" 'Online! July 2000 (2000-07) , 28TH ANNUAL AMERICAN SOCIETY FOR PHOTOBIOLOGY MEETING , SAN FRANCISCO XP002209526 Retrieved from the Internet: <URL: http://www.pol-us.net/ASP_Home/Meetings/Annual2000/28asp00.html> 'retrieved on 2002-07-31! Poster Session 2, Monday, 3. July 2000, 1:30 P.M., abstract number 388	1-30
X	US 3 579 339 A (CHANG CATHERINE THE-LIN ET AL) 18 May 1971 (1971-05-18) columns 4/5 procedures C, D, H, I column 8	1-3,14, 15,18, 26-30
X	DATABASE CA 'Online! CHEMICAL ABSTRACTS SERVICE, COLUMBUS, OHIO, US; SUZUKI, AKIRA ET AL: "Photosensitive color imaging materials" retrieved from STN Database accession no. 89:120931 XP002209527 abstract compound with CAS-RN 67389-41-7 & JP 53 009519 A (RICOH CO., LTD., JAPAN) 28 January 1978 (1978-01-28)	1-3,18, 26-30
X	TAYLOR, K. B.: "Chromatographic Separation and isolation of Metachromatic Thiazine Dyes" THE JOURNAL OF HISTOCHEMISTRY AND CYTOCHEMISTRY, vol. 8, 1960, pages 248-257, XP001098091 baltimore page 256; table 1	1-3,18, 26-30
X	STREKOWSKI L ET AL: "A SYNTHETIC ROUTE TO 3-(DIALKYLAMINO)PHENOTHIAZIN-5-IUM SALTS AND 3,7-DISUBSTITUTED DERIVATIVES CONTAINING TWO DIFFERENT AMINO GROUPS" JOURNAL OF HETEROCYCLIC CHEMISTRY, HETEROCORPORATION. PROVO, US, vol. 30, no. 4, 1 December 1993 (1993-12-01), pages 1693-1695, XP000196858 ISSN: 0022-152X examples 7-9	1,2,18, 26-30

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	US 3 641 016 A (KOROSI JENO ET AL) 8 February 1972 (1972-02-08) column 2 structure (I) examples ---	1,2,8, 10, 18-22, 26-30
X	JP 08 305026 A (FUJI PHOTO FILM CO LTD) 22 November 1996 (1996-11-22) page 12; examples SD-2 ---	1,2,18, 26-30
X	US 5 220 009 A (MAZUR YEHUDA ET AL) 15 June 1993 (1993-06-15) column 4/5 structure VII example 6 ---	1,2,18, 26-30
X	RODGERS, MICHAEL A. J. ET AL: "A laser flash kinetic spectrophotometric examination of the dynamics of singlet oxygen in unilammellar vesicles" PHOTOCHEM. PHOTOBIOL. (1982), 35(4), 473-7, XP001094399 page 474, column 1 compound "tetrol methylene blue" ---	1,2,18, 26-30
X	EP 0 510 668 A (TAKEDA CHEMICAL INDUSTRIES LTD) 28 October 1992 (1992-10-28) page 3, line 25 - line 40 page 11, line 27 -page 12, line 9 ---	1,2,7,8, 10, 13-15, 18-23,26
X	WAGNER S J ET AL: "FACTORS AFFECTING VIRUS PHOTOINACTIVATION BY A SERIES OF PHENOTHIAZINE DYES" PHOTOCHEMISTRY AND PHOTOBIOLOGY, OXFORD, GB, vol. 67, no. 3, May 1998 (1998-05), pages 343-349, XP001014580 ISSN: 0031-8655 page 344; figure 1 page 348, column 1 ---	1-4,8, 11-15, 18-23, 26-30
X	WAINWRIGHT M ET AL: "PHOTOBACTERICIDAL ACTIVITY OF PHENOTHIAZINIUM DYES AGAINST METHICILLIN-RESISTANT STRAINS OF STAPHYLOCOCCUS AUREUS" FEMS MICROBIOLOGY LETTERS, AMSTERDAM, NL, vol. 160, no. 2, 15 March 1998 (1998-03-15), pages 177-181, XP001042387 ISSN: 0378-1097 page 179; table 1 page 180 --- -/--	1-6, 8-11, 14-30

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MOURA, JOAO C. V. ET AL: "Synthesis and evaluation of phenothiazine singlet oxygen sensitizing dyes for application in cancer phototherapy" PHOSPHORUS, SULFUR AND SILICON AND THE RELATED ELEMENTS (1997), 120 & 121, 459-460 , XP001095339 page 459; table -----	1-3, 7-10, 18-23, 26-30

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 1-30 (partly)

Claims 1-3 encompass a large number of known compounds. The initial phase of the search revealed a very large number of documents relevant to the issue of novelty. So many documents were retrieved that it is impossible to determine which parts of the claim(s) may be said to define subject-matter for which protection might legitimately be sought (Article 6 PCT). For these reasons, a meaningful search over the whole breadth of the claim(s) is impossible. Consequently, the search can only be said to be complete for the compounds of claim 3, the example compounds mentioned in the description and the parts of claims 4-30 relating to these compounds (as far as they are clear, see below). Only representative documents have been cited for the other parts of the claims.

Claims 14-18 are unclear in scope (Art. 6 PCT) because they do not specify which structural parts of the compounds according to claims 1-3 are retained in the polymer, so that also polymers (or other products, cf. claim 16) are encompassed which have been prepared from unspecified starting materials not included in claims 1-3. A meaningful search and comparison with the prior art is thus impossible. The search has been restricted to products which are mentioned in the prior art in connection with phenothiazine compounds structurally defined in the claims (cf. also limitation above).

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 19-26 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [X] Claims Nos.: 1-30 (partly)
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

see FURTHER INFORMATION sheet PCT/ISA/210

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

PCT/GB 02/02278

Patent document cited in search report		Publication date	Patent family member(s)			Publication date
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			DE	1769359	A1	25-11-1971
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